

# Analysis of Cigarette-Smoke-Induced DNA Adducts by Butanol Extraction and Nuclease P1-Enhanced $^{32}\text{P}$ -Postlabeling in Human Lymphocytes and Granulocytes

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In an earlier study, we analyzed the aromatic DNA adducts separated from lymphocytes and granulocytes of smokers and nonsmokers using the nuclease P1-enhanced  $^{32}\text{P}$ -postlabeling assay. Here we compare the butanol extraction and nuclease P1-enhanced procedure on the same kind of samples. The DNA adducts of 42 per  $10^8$  nucleotides from smokers' lymphocytes were statistically higher ( $p < 0.05$ ) than those of 11 from nonsmokers', when analyzed by the nuclease P1 treatment, but not by the 1-butanol extraction. The radioactivity obtained from the DNA digests on the TLC plates was lower in butanol-treated DNA samples when compared to those of nuclease P1 digestion. Lymphocytes appear to be a suitable test tissue for determining aromatic carcinogen exposure when detecting smoking-related DNA adducts by the nuclease P1-enhanced  $^{32}\text{P}$ -postlabeling analysis.

## Introduction

Carcinogen-DNA adducts are currently considered a useful biological marker in risk assessment for potential environmental carcinogens (1). Cigarette smoking is a major cause of lung cancer and other diseases, and it is also an important source of exposure to polycyclic aromatic hydrocarbons (PAHs), aromatic amines, and tobacco-specific nitrosoamines (2-4). Carcinogenic PAHs of cigarette smoke were analyzed by the  $^{32}\text{P}$ -postlabeling assay from various human tissues, and the complexity of the adduct formation was shown in target and nontarget tissues (5-9). Although the chromatographic profiles obtained by the  $^{32}\text{P}$ -postlabeling technique were characteristic for aromatic compounds [benzo[a]pyrene; (8,10)], their identification is only well verified in animals (11). In an earlier study we separated lymphocytes and granulocytes of smokers and nonsmokers and analyzed aromatic DNA adducts by the nuclease P1-enhanced  $^{32}\text{P}$ -postlabeling assay (12). This study was carried out with the same lymphocyte and granulocyte DNA samples to investigate whether there are differences between the butanol extraction and the nuclease P1 enhancement procedures before

$^{32}\text{P}$ -postlabeling in determining the smoking-related adducts.

## Materials and Methods

Smokers and controls, blood sampling, DNA isolation, nuclease P1-enhanced  $^{32}\text{P}$ -postlabeling analysis, solvents used for the chromatographic purification, and the quantification of adduct levels are described elsewhere (12). Four micrograms of lymphocyte DNA from 10 smokers and 8 nonsmokers and granulocyte DNA from 10 smokers and from 9 nonsmokers were treated with the micrococcal nuclease (MN; 0.16 U) and spleen phosphodiesterase (SPD; 1.6  $\mu\text{g}$ ) at 37°C for 3.5 hr and treated with the nuclease P1 (3  $\mu\text{g}$  at 37°C for 40 min) according to the method of Reddy and Randerath (13). Butanol extraction was carried out according to the procedure of Gupta (14). After the incubation with MN and SPD, the DNA digests were diluted to 40  $\mu\text{L}$  by adding water (DNA concentration 0.1  $\mu\text{g}/\mu\text{L}$ ). For the extraction of adducts, 5  $\mu\text{L}$  of 10 mM tetrabutylammonium chloride (TBA), 5  $\mu\text{L}$  of 100 mM ammonium formate, pH 3.5, and 80  $\mu\text{L}$  water were added (total volume 130  $\mu\text{L}$ ). The mixture was extracted two times with water-saturated 1-butanol (130  $\mu\text{L}$ ) by mixing in a vortex 30 sec. The phases were separated by centrifuging for 30 sec in a microcentrifuge, and the butanol phases were combined (total volume about 260  $\mu\text{L}$ ). The butanol extract, first back-extracted two times with the 1-butanol-saturated water, was adjusted with 2.5  $\mu\text{L}$  of

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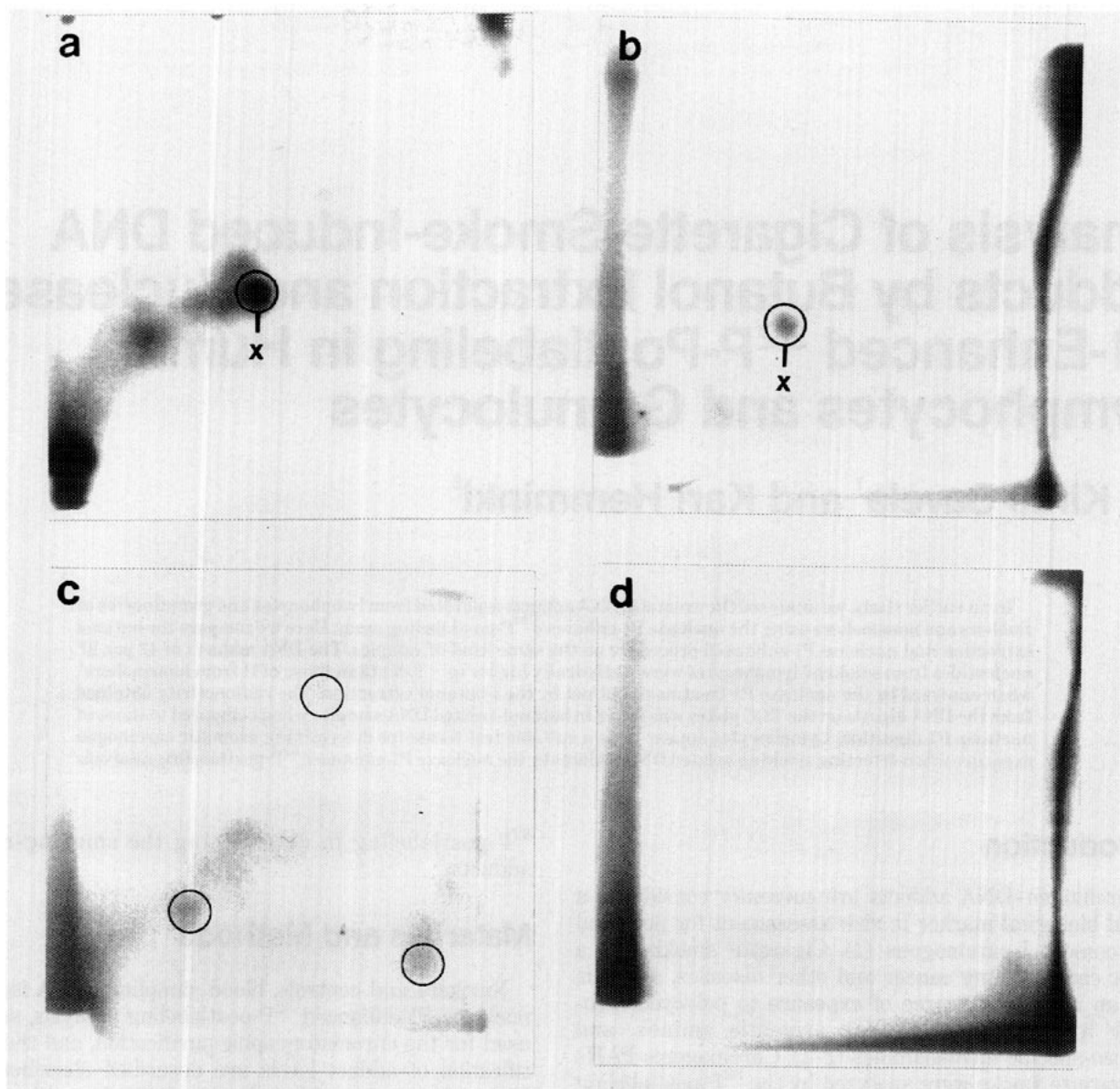


FIGURE 1. Autoradiograms of  $^{32}\text{P}$ -labeled DNA adducts from lymphocytes and granulocytes of smoker. Lymphocyte DNA of male smokers analyzed by (a,b) butanol extraction and (c,d) nuclease P1 treatment. Autoradiography was at  $-70^\circ\text{C}$  for 3.5 days. Circles marked "x" on panels a and b are the background spots, and the three open circles on panel c are smoking-specific adducts also obtained from the samples in a previous study (12).

200 mM Tris-HCl to pH 9.5 and evaporated in Speed-Vac (Savant Instruments Inc.). Calf thymus DNA ( $4\ \mu\text{g}$ ) and 7*R*,8*S*,9*S*-trihydroxy-10*R*-( $\text{N}^2$ -deoxyguanosyl)-3'-monophosphate)7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDG-dG; Midwest Research Institute, Kansas City, MO) was used as a reference compound in the labeling analysis.  $^{32}\text{P}$ -postlabeling was carried out for the DNA samples two to three times with  $30\ \mu\text{Ci}$  of commercially prepared [ $\gamma$ - $^{32}\text{P}$ ]ATP (10 pmole; 3000 Ci/mmole; Amersham), 33 pmole of cold ATP and 4 U of T4 polynucleotide kinase in the kinase buffer, pH 9.8, for 40 min at  $37^\circ\text{C}$  as detailed by Savela and Hemminki (12).

## Results

Figure 1 shows the autoradiograms obtained by the butanol- and nuclease P1-enriched  $^{32}\text{P}$ -postlabeling analysis from the same male smokers' lymphocyte and granulocyte DNA, respectively. Lymphocyte DNA obtained by butanol extraction showed a radioactive zone going up to the right corner, consisting of several faint spots (Fig. 1a). The granulocyte DNA of the same smoker showed only few radioactive spots on the autoradiograms when DNA was analyzed by the butanol extraction (Fig. 1b). The intensity of radioactivity was higher in smokers' lympho-

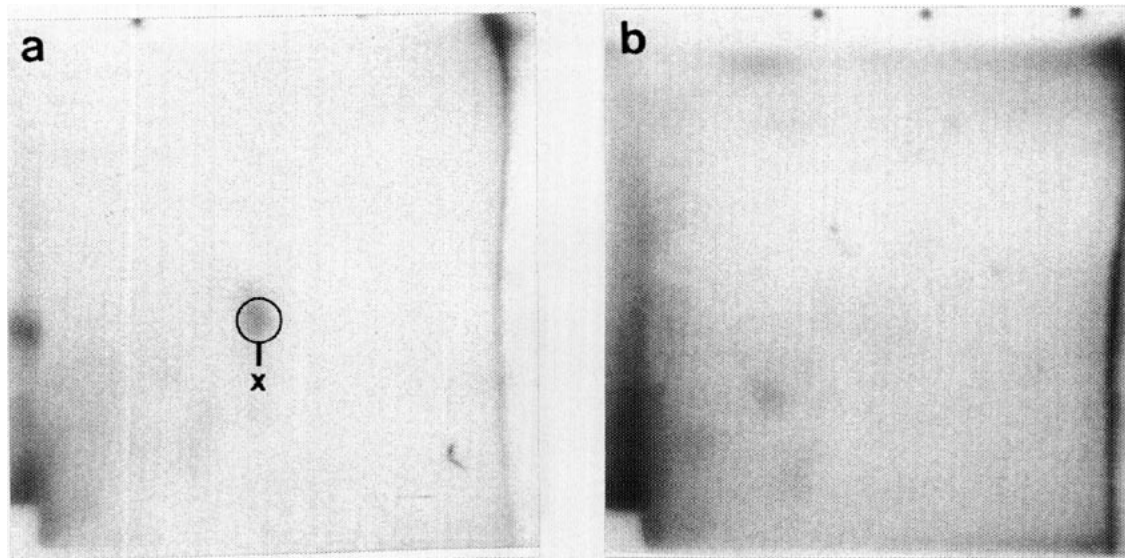


FIGURE 2.  $^{32}\text{P}$ -labeled calf thymus DNA analyzed by (a) butanol and (b) nuclease P1 treatment. Circle marked by "x" is the background spot obtained by the butanol extraction. Autoradiography was at  $-70^\circ\text{C}$  for 3.5 days.

cyte DNA analyzed by the nuclease P1 treatment (Fig. 1c) compared to that analyzed by the butanol extraction (Fig. 1a). Figure 1c shows the smoking-specific adducts detected in the previous study also (12). The radioactivity was very low on the autoradiograms obtained from granulocyte DNA of smokers by nuclease P1 enhanced  $^{32}\text{P}$ -postlabeling (Fig. 1d). A circle marked as an x-spot in the middle of the autoradiograms from smoker's lymphocytes indicates the background spot (Fig. 1a,b). These x-spots were also obtained when calf thymus DNA was analyzed by the butanol extraction procedure (Fig. 2a), but not by the nuclease P1 treatment (Fig. 2b).

The intensity of the spots on the autoradiograms obtained from the lymphocyte and granulocyte DNA of nonsmokers showed very low radioactivity when treated both with nuclease P1 and butanol extraction before  $^{32}\text{P}$ -postlabeling (Fig. 3a-d). Nonsmokers' lymphocyte DNA (Fig. 3a), but not granulocyte DNA, also showed the similar background x-spot that was obtained from smokers' lymphocyte and granulocyte DNA extracted by butanol (Fig. 1a,b).

The levels of adducts ranged from 4 to 32 per  $10^8$  nucleotides in lymphocytes of both smokers and nonsmokers after butanol extraction and from 16 to 98 in smokers and from 15 to 28 in nonsmokers when analyzed by nuclease P1-enhanced  $^{32}\text{P}$ -postlabeling (Table 1). The levels of adducts were low in granulocytes, ranging from 4 to 13 per  $10^8$  nucleotides in smokers and from 3 to 13 in nonsmokers after butanol extraction, and from 4 to 14 in smokers and from 5 to 19 in nonsmokers after nuclease P1-enhanced procedure (Table 1). The mean number of DNA adducts/ $10^8$  nucleotides detected from smokers' and nonsmokers' lymphocytes and granulocytes are presented in Figure 4. The error bars indicate the standard deviation of

the two to three mean parallel  $^{32}\text{P}$ -postlabeled experiments. The total DNA adduct levels of  $42 \pm 7.5/10^8$  nucleotides from smokers' lymphocytes were significantly higher than those of  $22 \pm 1.9/10^8$  nucleotides from nonsmokers, when analyzed by the nuclease P1-enhanced postlabeling assay (Table 2). The butanol extraction analysis yielded from the smokers' lymphocytes  $20 \pm 7.5$  and from those nonsmokers' lymphocytes  $13 \pm 3.1$  DNA adducts per  $10^8$  nucleotides (Table 1). The total DNA adduct levels in granulocytes, when analyzed by both the butanol extraction and nuclease P1-enhanced  $^{32}\text{P}$ -postlabeling, were statistically lower ( $p < 0.05$ ) compared to those of lymphocytes, but showed no statistically significant difference between smokers' and nonsmokers' granulocyte DNA adduct levels (Table 2).

Figure 5 shows the comparison of DNA adduct levels from the lymphocytes of 10 smokers when analyzed by nuclease P1-enhanced  $^{32}\text{P}$ -postlabeling in years 1990 and 1991. The relationship of the adduct levels was relatively good, although not statistically significant ( $y = 0.74x + 20.5$ ,  $r = 0.55$ ,  $n = 10$ ).

## Discussion

As the nuclease P1 enzyme is thought to dephosphorylate the adducted mononucleotides formed by tobacco-specific nitrosoamines and aromatic amines bound to the C-8 position of guanine, butanol extraction is an alternative method of detecting adducts formed by cigarette smoke (14-16). When butanol extraction was applied to the DNA samples, all backgrounds of the TLC plates were clean (Fig. 1a,b compared to c,d), suggesting that butanol did not extract some minor adducts. Overall, lower DNA adduct levels were obtained from smokers' lymphocytes

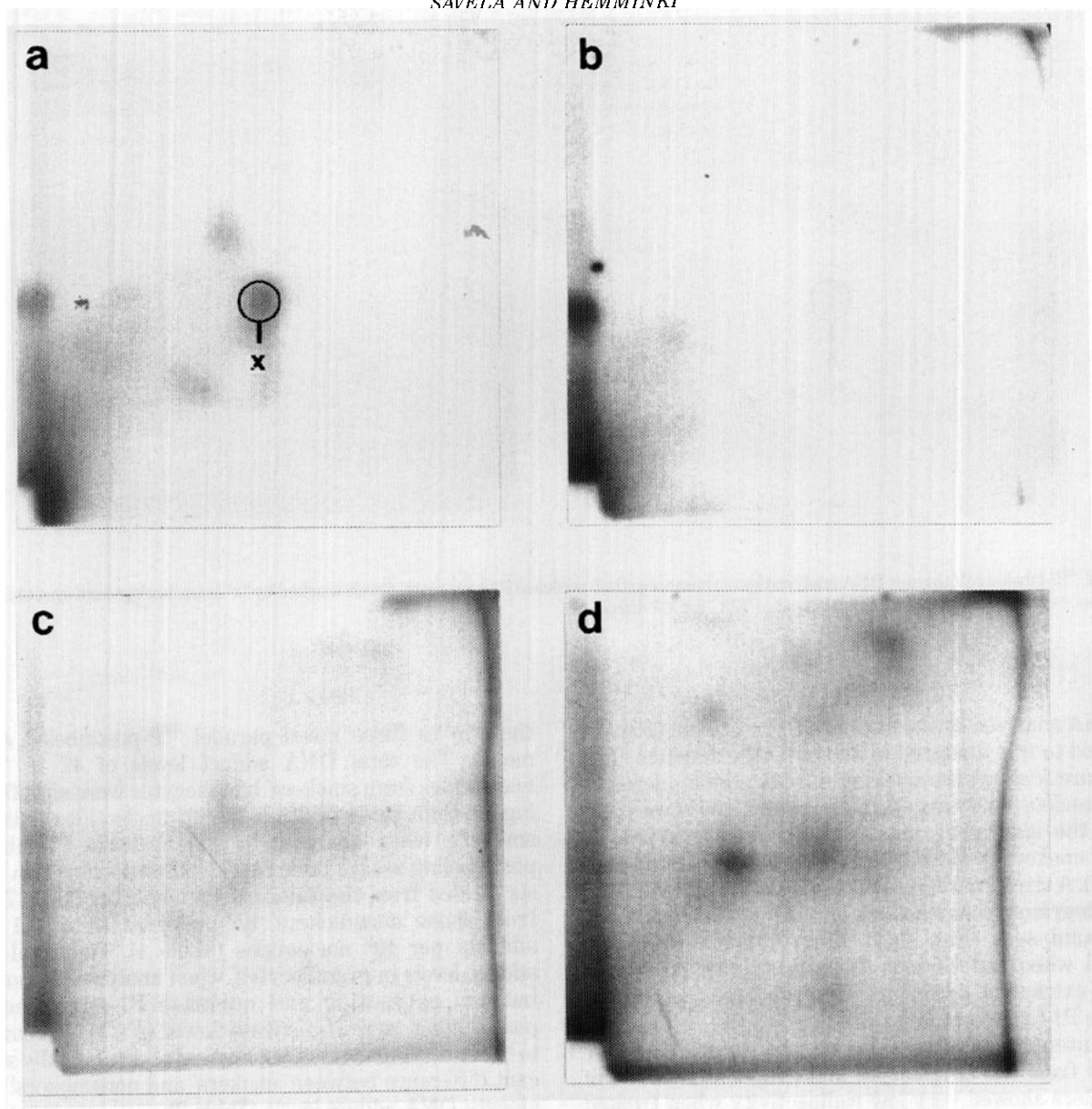


FIGURE 3. Autoradiograms obtained by the  $^{32}\text{P}$ -labeling of DNA from lymphocyte and granulocyte of male nonsmoker. Lymphocyte DNA of a nonsmoker analyzed by (a,b) butanol extraction and (c,d) nuclease P1 treatment. Autoradiography was at  $-70^{\circ}\text{C}$  for 3.5 days.

and granulocytes by the butanol extraction than by the nuclease P1 treatment, perhaps suggesting lower levels of P1-sensitive nitroaromatic and aromatic amine DNA adducts than stable PAH-type adducts (16). Possible reasons for lower adduct levels could also be the loss of material during the butanol extraction steps and discriminating any adducted dinucleotides. Yet, as long as the  $^{32}\text{P}$ -postlabeled adducts remain unidentified, it is difficult to assess the selectivity of the two methods (14,16,17).

A statistically significant difference was obtained between the total DNA adduct levels between the smokers' and nonsmokers' lymphocytes analyzed by the nuclease P1 procedure only (Table 2). The relationship of the adduct data from smokers' lymphocytes was studied, with the

results obtained with the same samples in 1990 (Fig. 5). The results of this study confirm the same levels of adducts determined by the nuclease P1-enhanced  $^{32}\text{P}$ -postlabeling analysis in the 2 years (12). The adduct data are also in accordance, irrespective of the pretreatment, with the higher DNA adduct levels in lymphocytes than in granulocytes of smokers. The  $^{32}\text{P}$ -postlabeled total white blood cell DNA adducts has been used in several occupational studies as an indicator of PAH exposure. By contrast, no effect of smoking was seen in these studies (18-20). The human lymphocytes are thus more suitable for use in determining the effect of smoking or, perhaps, in humans occupationally or environmentally exposed to PAHs (12,21).

**Table 1. Summary data of DNA adducts analyzed by the butanol extraction and nuclease P1 enhanced  $^{32}\text{P}$ -postlabeling.**

Subjects	Adducts/ $10^8$ nucleotides $\pm$ SE			
	Butanol extraction		Nuclease P1 treatment	
	Lymphocytes	Granulocytes	Lymphocytes	Granulocytes
Smokers				
1	32 $\pm$ 14	13 $\pm$ 5.6	34 $\pm$ 7.5	8.7 $\pm$ 3.4
2	24 $\pm$ 0.5	10 $\pm$ 5.7	57 <sup>a</sup>	11 $\pm$ 5.3
3	24 <sup>a</sup>	5 $\pm$ 0.0	98 <sup>a</sup>	8.0 $\pm$ 0.9
4	25 $\pm$ 5.0	6.8 $\pm$ 1.3	45 $\pm$ 9.0	9.7 $\pm$ 6.3
5	21 $\pm$ 11	8.0 $\pm$ 0.2	38 $\pm$ 18	4.2 $\pm$ 1.5
6	30 $\pm$ 10	12 $\pm$ 2.6	14 $\pm$ 1.9	12 $\pm$ 3.4
7	8.0 $\pm$ 2.9	8.9 $\pm$ 1.8	35 $\pm$ 19	14 $\pm$ 2.5
8	22 $\pm$ 6.2	11 $\pm$ 6.9	33 $\pm$ 10	7.8 $\pm$ 2.3
9	3.9 $\pm$ 0.5	5.9 $\pm$ 1.5	16 $\pm$ 4.6	11 $\pm$ 3.5
10	7.0 $\pm$ 1.4	4.1 $\pm$ 1.3	48 $\pm$ 25	10 $\pm$ 3.5
Nonsmokers				
1	20 $\pm$ 4.0	5.6 $\pm$ 0.6	23 $\pm$ 1.5	10 $\pm$ 7.0
2	32 $\pm$ 6.0	3.4 $\pm$ 1.2	21 $\pm$ 0.5	4.9 $\pm$ 1.5
3	10 $\pm$ 0.6	4.8 $\pm$ 1.8	27 $\pm$ 1.0	12 $\pm$ 7.9
4	—	8.1 $\pm$ 1.9	—	19 $\pm$ 3.5
5	9.9 $\pm$ 4.1	11 $\pm$ 2.7	28 $\pm$ 7.6	7.2 $\pm$ 0.8
6	11 $\pm$ 3.8	8.0 $\pm$ 3.8	18 $\pm$ 7.4	9.7 $\pm$ 5.4
7	8.0 $\pm$ 3.6	13 $\pm$ 5.3	15 $\pm$ 3.2	13 $\pm$ 2.7
8	11 $\pm$ 2.2	7.4 $\pm$ 2.4	28 $\pm$ 6.4	14 $\pm$ 3.4
9	4.5 $\pm$ 1.5	6.1 $\pm$ 0.6	15 $\pm$ 4.8	8.6 $\pm$ 1.4

<sup>a</sup>Only one postlabeling experiment. Otherwise, adduct levels are given as a mean number of adducts of two to three  $^{32}\text{P}$ -postlabelings.

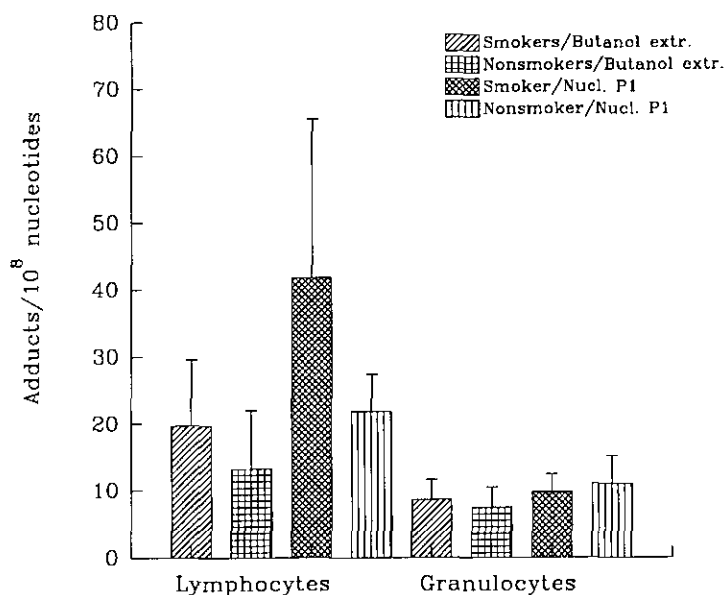


FIGURE 4. Bar chart of the mean adduct levels obtained from lymphocytes and granulocytes of smokers and nonsmokers analyzed by butanol and nuclease P1-enhanced  $^{32}\text{P}$ -postlabeling.

**Table 2. DNA adducts analyzed from lymphocytes and granulocytes of smokers and nonsmokers by butanol extraction and nuclease P1 enhanced  $^{32}\text{P}$ -postlabeling assay.**

Method	Adducts/ $10^8$ nucleotides $\pm$ SE			
	Smokers		Nonsmokers	
	Lymphocytes	Granulocytes	Lymphocytes	Granulocytes
Butanol extraction	20 $\pm$ 3.1	8.6 $\pm$ 0.9	13 $\pm$ 3.1	7.4 $\pm$ 1.1
Nuclease P1 treatment	42 $\pm$ 7.5*	9.7 $\pm$ 0.9	22 $\pm$ 1.9	11 $\pm$ 1.4

\* $P < 0.05$  analyzed by two-tailed Student's  $t$ -test;  $t$ -value between DNA adducts of smokers' and nonsmokers' lymphocytes is 2.3, when analyzed by nuclease P1 enhanced  $^{32}\text{P}$ -postlabeling.

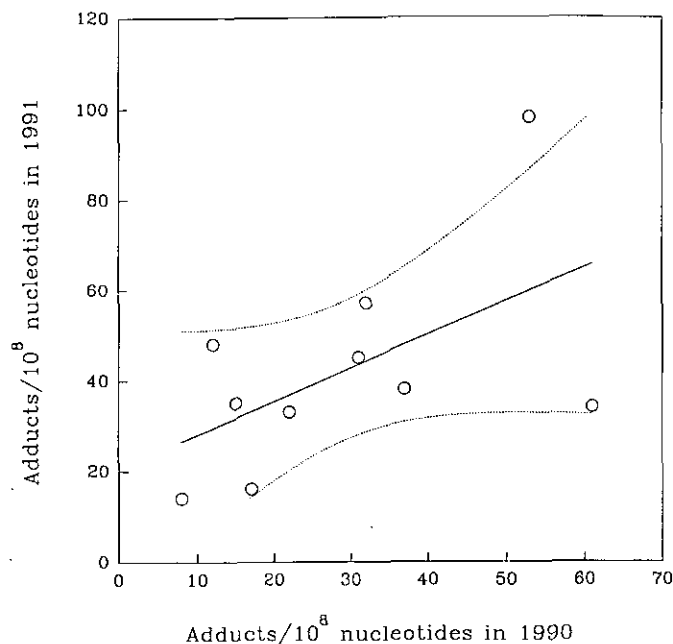


FIGURE 5. Relationship between the DNA adducts/10<sup>8</sup> nucleotides analyzed from smokers' lymphocytes in 1990 and 1 year later. The pair of dotted lines represent the 95% confidence limit. Degrees of freedom = 8, correlation coefficients = 0.55,  $p = 0.07$ .

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